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Overexpression of monoubiquitin improves photosynthesis in transgenic tobacco plants following high temperature stress

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ABSTRACT

The ubiquitin/26S proteasome system (Ub/26S) is implicated in abiotic stress responses in plants. In this paper, transgenic tobacco plants overexpressing *Ta-Ub2* from wheat were used to study the functions of Ub in the improvement of photosynthesis under high temperature (45 °C) stress. We observed higher levels of Ub conjugates in transgenic plants under high temperature stress conditions compared to wild type (WT) as a result of the constitutive overexpression of *Ta-Ub2*, suggesting increased protein degradation by the 26S proteasome system under high temperature stress. Overexpressing Ub increased the photosynthetic rate (P_n) of transgenic tobacco plants, consistent with the improved ATPase activity in the thylakoid membrane and enhanced efficiency of PSII photochemistry. The higher D1 protein levels following high temperature stress in transgenic plants than WT were also observed. These findings imply that Ub may be involved in tolerance of photosynthesis to high temperature stress in plants. Compared with WT, the transgenic plants showed lower protein carbonylation and malondialdehyde (MDA) levels, less reactive oxygen species (ROS) accumulation, but higher antioxidant enzyme activity under high temperature stress. These findings suggest that the improved antioxidant capacity of transgenic plants may be one of the most important mechanisms underlying Ub-regulated high temperature tolerance.

1. Introduction

Temperature is one of the major environmental factors affecting plant growth, development and also sensitive with photosynthetic apparatus. Heat stress due to increased temperature is an agricultural problem in many areas of the world. Transitory or constantly high temperatures cause an array of physiological and biochemical changes in plants, which can affect plant growth. Following temperature–stress, the loss of photosynthetic electron transport in plant was attributed to the thermolability of PSII that may cause the generation of $^1O_2$, which is potentially dangerous [1]. Plant responses to environmental factors have often been associated with active oxygen species (ROS), including hydroxyl radicals and H_2O_2 [2]. Excess production of ROS causes oxidative damage of cellular compartments under biotic and abiotic stresses [3].

When the photon energy is in excess, photosystem II (PSII) is the primary target for photoinhibition, which is strictly related to the rate of electron flow from PSII [3]. D1 protein, one of the proteins of PSII reaction center complex with rapid turnover, could be used to reflect the degree of photoinhibition of PSII [4]. Some previously studies showed that singlet oxygen is able to cause oxidative damage in PSII directly [5,6]. The repair of PSII is triggered by the degradation of the photo-damaged D1 protein, which is subsequently replaced with newly synthesized protein.

Plants have evolved a great diversity of mechanisms for surviving exposure to above optimal temperatures, including protein synthesis and degradation. Previous reports have suggested that the ubiquitin/26S proteasome system (Ub/26S), which mediates a post-translational modification of cellular proteins, commonly targeting them for rapid degradation by the proteasome [7–9]. Exposure to high temperature stress affects the stability of many cellular proteins that might become targets for degradation by Ub/26S. In this system, misfolded proteins are marked with

Abbreviations: APX, ascorbate peroxidase; CAT, catalase; C_i, intercellular CO_2 concentration; E, transpiration rate; F_v/F_m, maximal efficiency of PSII photochemistry; g_s, stomata conductance; MDA, malondialdehyde; P_n, photosynthetic rate; POD, peroxidase; Φ_{PSII}, actual efficiency of PSII; ROS, reactive oxygen species; SOD, superoxide dismutase; Ub, ubiquitin; WT, wild-type.

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polyubiquitin chains by an E2 ubiquitin-conjugating enzyme and an E3 ubiquitin ligase [10–13]. The tagged proteins are subsequently degraded into small polypeptides by the 26S proteasome [14,15].

As the name implies, ubiquitin (Ub) is nearly ubiquitous, being present in all eukaryotic species examined and all tissues of plants [7,16]. In Ub/26S pathway, Ub is a small (76 amino acids) multifunctional protein [17], and one of its primary functions is to tag proteins for selective degradation by the 26S proteasome [18,19]. Ub expression can be induced by various stresses in plants [19,20]. However, limited studies have been carried out to examine the effects of altered Ub gene expression on high temperature tolerance in plants.

In our previous work, a monoubiquitin gene *Ta-Ub2* from wheat (*Triticum aestivum* L.) was cloned and expressed in the sense orientation in transgenic tobacco. We found that overexpression of *Ta-Ub2* improved the drought tolerance and salt resistance of transgenic tobacco plants [21,22]. In the present study, we investigated the response of several photosynthetic parameters to high temperature stress, comparing the transgenic plants with wild type (WT). Our results suggest that overexpression of the *Ta-Ub2* gene increased the tolerance of leaf photosynthesis and the stability of thylakoid membrane protein complexes under high temperature stress. The improved antioxidative ability may be involved in this improvement.

2. Materials and methods

2.1. Plant materials and growth conditions

The sense *Ta-Ub2* transgenic tobacco (*Nicotiana tabacum*) lines (T_2-2, T_2-11 and T_2-13) and wild type (WT) were used in this study. *Ta-Ub2*, a cDNA sequence containing a single Ub repeat and a 3′ non-coding region of a polyubiquitin gene, was isolated from wheat (*T. aestivum* L.) by reverse transcription-polymerase chain reaction (RT-PCR). The transgenic plants carrying the recombinant construct of *Ta-Ub2* gene under the control of CaMV 35S promoter were produced in our Lab [21]. The CaMV 35S promoter is one of the most widely used promoters because it exhibits a high level of transcriptional activity in a variety of plant tissues. In our previous papers, we used both WT and T-GUS (vector control, carrying the recombinant construct of the beta-glucuronidase (pBI-GUS) gene alone under control of the CaMV 35S promoter and the nopaline synthase 3′ termination sequences in the sense orientation) as two controls [21,22]. There are no significant differences between T-GUS and WT, so in this paper, we only use WT as a control. The sense transgenic plants were T_2 homozygous generation. The expressed level of the heterogeneous gene was identified using the polymerase chain reaction (PCR) as our previous paper [21]. In the supplemental experiments, the antisense *Nt-Ub1* transgenic tobacco plants with inhibited Ub expression [21] were also used.

For full-grown tobacco plants, seeds were sown in pots (8 cm × 10 cm) containing vermiculite soaked with half-strength Hoagland nutrient solution (the irrigation was enough to avoid water stress and maintained the natural growth) and grown in a chamber at 25 °C with a 16/8 h (light/dark) cycle (300–400 μmol photons m^{-2} s^{-1}) and a relative humidity of 75–80%. High temperature stress was performed on 4-month-old tobacco plants with 7–8 leaves. The whole plants were exposed to high temperature (45 °C) for 3, 6 and 9 h only under the light. The actual leaf temperature was almost the same as that in the environment of chamber during measurement.

2.2. SDS-PAGE and immunological analyses of Ub

Total protein was extracted from tobacco leaves. Protein content was determined by the dye-binding assay according to Bradford [23]. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% and 14% two concentration gel and transferred to a polyvinylidene fluoride membrane (Millipore, Saint-Quentin, France). Proteins were routinely detected with the Ub antibody (Sigma). Quantitative analysis was performed using the Tanon GIS system (Tanon, Shanghai, China).

2.3. Measurement of photosynthetic gas exchange and chlorophyll fluorescence parameters

The full-grown tobacco plants were used to estimate the net photosynthetic rate (P_n), transpiration rate (E), Intercellular CO_2 concentration (C_i) and stomata conductance (g_s) with a portable photosynthetic system (CIRAS-2, PP Systems, Hitchin, UK). The measurements were conducted at a CO_2 concentration of 360 μl l^{-1}, PFD of 800 μmol m^{-2} s^{-1} and relative humidity of 60–70% and the temperature inside the leaf chamber was 25 °C. Before measurement, plants were kept at 25 °C, 100 μmol m^{-2} s^{-1} PFD for 30 min to induce the stomata to open, and then illuminated at PFD of 800 μmol m^{-2} s^{-1} for 15 min to be acclimated.

The maximal efficiency of PSII photochemistry (F_v/F_m) and the actual efficiency of PSII (Φ_{PSII}) of the same tobacco leaves were measured with a portable pulse-modulated fluorometer FMS-2 (Hansatech Instruments, UK). For quenching analyses, the leaves were illuminated with actinic light intensities of 800 μmol m^{-2} s^{-1} for 30 min, which was sufficient for the induction of steady state light conditions. Plants were acclimated in darkness for 30 min before F_v/F_m measurement [24].

2.4. Determinations of thylakoid membrane Ca^{2+}-ATPase and Mg^{2+}-ATPase activities

Ca^{2+}-ATPase activity was determined following the activation of the coupling factor by trypsin according to Huang [25]. The chloroplast suspension (1 ml) was added to 1 ml of a medium containing 250 mM Tricine (pH 8.0), 20 mM ethylenediamine tetraacetic acid (EDTA), 10 mM ATP, and 2 mg cm^{-3} trypsin, and incubated at 20 °C for 10 min. Then, 0.1 ml bovine serum albumin (10 mg l^{-1}) was added to the mixture to terminate the reaction. Next, 0.5 ml of the incubated chloroplast suspension was added to 0.5 ml of the reaction mixture containing 500 mM Tricine (pH 8.0), 10 mM ATP, and 50 mM $CaCl_2$. The mixture was incubated at 37 °C for 10 min and centrifuged at 3000 *g* for 1 min. Finally, ferrous sulfate ammonium molybdate was added to the resulting supernatant, which was used for determination of inorganic phosphorus absorbance at 660 nm.

For the measurement of Mg^{2+}-ATPase activity, 1 ml of the chloroplast suspension was added to 1 ml of a medium containing 250 mM Tricine (pH 7.0), 500 mM NaCl, 50 mM $MgCl_2$, and 50 mM 1,4-dithiothreitol (DTT), and incubated in high irradiance at 25 °C for 5 min; 0.1 cm^3 bovine serum albumin (10 mg l^{-1}) was added to the mixture to terminate the reaction. The incubated chloroplast suspension (0.5 ml) was then added to the 0.5 ml reaction mixture containing 500 mM Tricine (pH 8.0), 50 mM ATP, and 50 mM $MgCl_2$. The reaction mixture was incubated at 37 °C for 10 min and centrifuged at 3000 *g* for 1 min. Finally, ferrous sulfate ammonium molybdate was added to the resulting supernatant, which was used for determination of inorganic phosphorus absorbance at 660 nm.

2.5. Detection of D1 protein by immunoblotting

Thylakoid membranes were prepared according to the method as described in [26]. The tobacco leaves were homogenized in an ice-cold isolation buffer containing 400 mM sucrose, 50 mM HEPES, pH 7.8, 10 mM NaCl, 2 mM EDTA and 2 mM $MgCl_2$ and filtered through three layers of pledget. The filtrate was centrifuged at